

Long noncoding RNA-LET, which is repressed by EZH2, inhibits cell proliferation and induces apoptosis of nasopharyngeal carcinoma cell

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Abstract Recent studies have reported that long noncoding RNAs (lncRNAs) play critical roles in carcinogenesis and progression. LncRNA-LET, a recently identified lncRNA, has been shown to be a tumor suppressor in hepatocellular carcinoma. However, the expression and functional of lncRNA-LET in other type of cancers remain largely unknown. In this study, we found that lncRNA-LET was significantly downregulated in nasopharyngeal carcinoma (NPC) tissues compared with corresponding normal tissues. Decreased LET expression is significantly correlated with advanced clinical stage, larger tumor size, increased lymph node tumor burden, and poor survival of NPC patients. Gain- and loss-of-function experiments demonstrated that enhanced LET expression inhibited NPC cells proliferation and induced cell apoptosis. By contrast, the knockdown of LET promoted NPC cells proliferation and inhibited cell apoptosis. Importantly, we found lncRNA-LET is transcriptional repressed by EZH2-mediated H3K27 histone methylation on the *LET* promoter. The expressions of EZH2 and lncRNA-LET are significantly inversely correlated in NPC tissues. Collectively, these findings indicate a pivotal role for lncRNA-LET in NPC cell proliferation and apoptosis, and reveal an epigenetic mechanism for lncRNA-LET dysregulation.

Qiuzhen Sun and Hongbing Liu contributed equally to this work and should be regarded as joint first authors.

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Introduction

Nasopharyngeal carcinoma (NPC), which arises from the nasopharyngeal epithelium, is one of the most common types of head and neck tumors [1–3]. It is vastly more prevalent in Southeast Asia, resulting in serious health problems in these regions [4]. Therefore, it is of great clinical value to further reveal the molecular mechanisms of NPC carcinogenesis, and develop effective therapeutic strategies [5–7].

Long noncoding RNA (lncRNA) is a novel type of RNA molecule, longer than 200 nucleotides in length, with no protein coding potential [8–10]. Recently, many studies have found lncRNAs are extensively transcribed from the genomic DNA [11–13]. They have been shown to be deregulated in many cancers and participate in carcinogenesis and cancer progression by modulating the expression of many oncogenes or tumor suppressor genes [14–16]. However, there are only several studies of lncRNAs in human NPC, and the expressions and functions of lncRNAs in NPC remain largely unknown [17–20]. LncRNA-LET, a recently identified lncRNA, has been reported to be downregulated and exhibit tumor-suppressive roles in hepatocellular carcinoma, cervical cancer, gastric cancer, and gallbladder cancer [21–24]. However, the expression, function, and the factors contributing to its abnormal expression remain unknown in human NPC.

In the present study, we investigated the expression levels of LET, its association with clinicopathological characteristics, and its biological functions in NPC cells. Our results show that LET is downregulated in NPC

tissues, and its decreased expression is associated with advanced clinical stage, larger tumor size, increased lymph node tumor burden, and poor prognosis of NPC patients. Overexpression of LET inhibited NPC cells proliferation and induced cell apoptosis, while the knockdown of LET promoted NPC cells proliferation and inhibited cell apoptosis. Moreover, we also found that enhancer of zeste homolog 2 (EZH2) repressed LET expression via H3K27 histone methylation on the *LET* promoter.

Materials and methods

Tissue samples

A total of 68 NPC tissues and their pair-matched adjacent normal tissues were obtained with informed consent from patients who had been diagnosed at the Second Affiliated Hospital of Nanchang University, Nanchang, China. These patients were subjected to biopsy before standard chemoradiotherapy, and the survival of these patients was followed up with a median follow-up period of 56 months. The clinical stages of these patients were classified according to American Joint Committee on Cancer (AJCC). The clinicopathological information of these patients is described in Table S1. This study was performed with the approval of the Research Ethics Committee of the Second Affiliated Hospital of Nanchang University.

Cell cultures

The normal immortalized human nasopharyngeal epithelial cell line NP69, and the human NPC cell lines CNE2 and HNE2 were obtained from Sun Yat-sen University, Guangzhou, China. The cells were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT, USA) and maintained at 37 °C in a humidified incubator containing 5 % CO₂.

RNA extraction and qPCR analyses

Total RNAs were extracted using TRIzol (Life Technologies) according to the manufacturer's protocol. First-strand cDNA was generated using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR was performed on ABI 7500 real-time PCR system (Life Technologies). For each sample, gene expression was normalized to the level of GAPDH. The primer sequences used in this study were as follows: *LET*, 5'-GGAGTAAAGGGAAAGAGTTGC-3' (forward) and 5'-GTGTCTGGACTGGCAAAT-3' (reverse); *EZH2*, 5'-CAGCCTTGACAGTTCGT-3' (forward) and 5'-AGATGGTGCCAGCAATAGA-3' (reverse);

GAPDH, 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse). The real-time PCRs were carried out in triplicate. The relative RNA expression was calculated using the comparative Ct method.

Vectors construction

The complementary DNA encoding *LET* or *EZH2* was PCR-amplified by the prime STAR HS DNA polymerase (Takara) and subcloned into the *Bam*H I and *Eco*R I, or *Kpn* I and *Bam*H I sites of pcDNA3.1 vector (Life Technologies), respectively. The primers used were as follows: 5'-CGGGATCCGAAAGAATGTGGCCCCAGAGG-3' (forward) and 5'-GGAATTCAGGAAGCAGCTAAACCTCAATCCTA-3' (reverse) for *LET*; 5'-GGGGTACCGAAGAATAATCATGGGCCAGACTGG-3' (forward) and 5'-CGGGATCCAGATGTCAAGGGATTTCCATTTCTC-3' (reverse) for *EZH2*.

Small interfering RNA synthesis and transfection

Small interfering RNAs (siRNAs) specifically targeting *LET* and *EZH2* were purchased from GenePharma company (Suzhou, China). The siRNA sequences were 5'-GTGCA TGTGGTAGGTTAGATT-3' for *LET*; 5'-AAGACTCTGAATGCAGTTGCT-3' for *EZH2*. Transfections were performed using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions.

Cell proliferation assay

A total of approximately 1000 transfected cells were plated in 96-well plates. Cell proliferation was assessed every 24 h using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbencies at each time point were used to plot cell proliferation curves. Ethynyl deoxyuridine (EdU) immunofluorescence staining was carried out using an EdU Kit (Roche, Mannheim, Germany). All experiments were performed in triplicate.

Western blotting

Protein extracts were prepared in a 1× sodium dodecyl sulfate buffer. Identical quantities of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After incubation with antibodies specific for cleaved caspase-3 (Cell Signaling Technology, Boston, USA) or β-actin (Sigma-Aldrich, Saint Louis, MO, USA), the blots were incubated with goat anti-rabbit or anti-mouse

secondary antibody (Cell Signaling Technology) and visualized with enhanced chemiluminescence.

TdT-mediated dUTP nick end labeling assay

TdT-mediated dUTP nick end labeling (TUNEL) assays were performed using the Dead EndTM Fluorometric TUNEL System (Promega, Madison, WI, USA) to measure the fragmented DNA of apoptotic cells according to the manufacturer's instructions. TUNEL-positive cells were defined as cells with nuclear green fluorescence labeling.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out using the EZ ChIPTM Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. An anti-EZH2 antibody (Millipore) or anti-H3K27me3 antibody (Millipore) was used to precipitate DNA-protein complexes. ChIP-derived DNA was quantified using qPCR. The primer sequences specific for the *LET* promoter were as follows: 5'-CCTTGG TTGGTCCCTTGA-3' (forward) and 5'-TTTCTGTA CGCCGGTCATG-3' (reverse).

Generation of cells stably overexpressing LET

To generate the cell line stably overexpressing LET, CNE2 cells were transfected with the plasmid pcDNA3.1-LET and selected with neomycin (800 µg/ml) for 4 weeks.

In vivo tumorigenesis assay

LET stably overexpressed CNE2 cells (1.0×10^6) were implanted subcutaneously into the flanks of nude mice. Primary tumor growth was detected by measuring the tumor length (*L*) and width (*W*), and tumor volume was calculated according to the equation $V = 0.5 \times LW^2$. The animal studies were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Nanchang University.

Statistical analysis

For comparisons, Wilcoxon signed-rank test, log-rank test, Pearson Chi-square test, Student's *t* test, and Pearson correlation analysis were carried out using the SPSS 18.0 software package (SPSS, Chicago, IL, USA). Differences were defined as statistically significant for *P* values <0.05.

Results

LET expression in NPC tissues and its association with clinicopathological characteristics

The expression level of LET was examined using qRT-PCR in 68 pairs of NPC tissues and pair-matched adjacent normal tissues. LET transcript expression was significantly downregulated in NPC tissues compared with corresponding normal tissues ($P < 0.001$, Fig. 1a).

We next examined the correlation between the expression of LET and clinicopathological characteristics of the 68 NPC samples. The 68 NPC samples were classified into two groups according to the median expression level of LET. Correlation regression analysis showed that decreased LET expression is significantly correlated with advanced clinical stage ($P = 0.026$), larger tumor size ($P = 0.008$), and increased lymph node tumor burden ($P = 0.049$; Table 1).

Next, we detected the expression of LET in immortalized normal nasopharyngeal epithelial cell line NP69 and NPC cell lines CNE2 and HNE2. LET is significantly downregulated in NPC cell lines, compared with normal nasopharyngeal epithelial cell line (Fig. 1b).

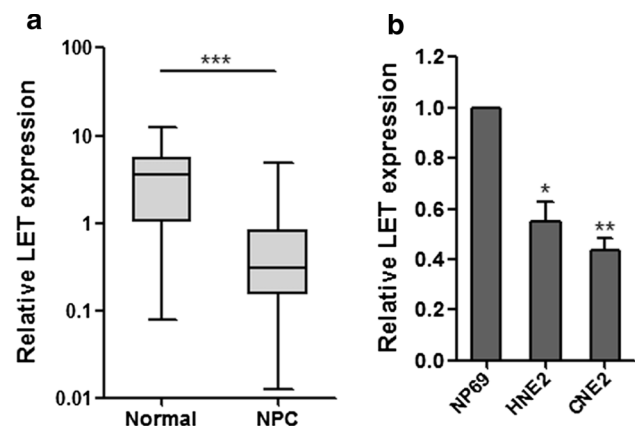


Fig. 1 LET expression is downregulated in NPC tissues and cell lines. **a** The expression level of LET was analyzed by qRT-PCR in 68 pairs of NPC tissues and pair-matched adjacent normal tissues. The horizontal lines in the box plots represent the medians, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. The significant differences between samples were analyzed using the Wilcoxon signed-rank test. *** $P < 0.001$. **b** LET expression level in immortalized normal nasopharyngeal epithelial cell line NP69 and NPC cell lines CNE2 and HNE2 was analyzed by qRT-PCR. Data are presented as the mean \pm SE based on at least three independent experiments. * $P < 0.05$; ** $P < 0.01$

Table 1 Association between LET expression and clinicopathological characteristics

Characteristics	Expression of LET		χ^2	P value
	Low	High		
Gender			1.722	0.189
Male	26	21		
Female	8	13		
Age			0.530	0.467
<48	18	15		
≥48	16	19		
Clinical stages			4.976	0.026
I–II	9	18		
III–IV	25	16		
Tumor size			7.124	0.008
T1–T2	11	22		
T3–T4	23	12		
N stages			3.886	0.049
N0	10	18		
N1–N3	24	16		
Metastasis			3.238	0.072
Yes	10	4		
No	24	30		

Low LET expression predicts poor prognosis of NPC patients

We further examined whether the LET expression level was correlated with the outcome of NPC patients. Kaplan–Meier survival analysis and log-rank tests showed that low LET expression in NPC tissues is significantly associated with poor recurrence-free survival ($P = 0.0151$) and overall survival ($P = 0.0214$; Fig. 2a, b). These results

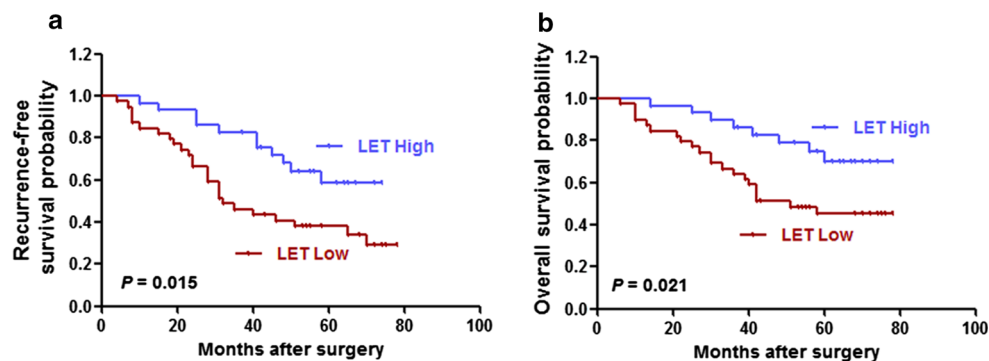


Fig. 2 Low LET expression predicts poor prognosis of NPC patients. **a** Kaplan–Meier analyses of correlations between the LET expression level and recurrence-free survival of 68 NPC patients. **b** Kaplan–Meier analyses of correlations between the LET expression level and

indicate that decreased expression of LET may play a pivotal role in the progression of NPC.

Overexpression of LET inhibits cell proliferation and induces apoptosis of NPC cell

Based on the results that LET is downregulated in NPC and associated with the progression of NPC, we next examined the biological function of LET in NPC. As shown in Fig. 3a, we enhanced LET expression in CNE2 cells by transfecting a LET expression vector (pcDNA3.1-LET). CCK-8 assays indicated that enhanced LET expression significantly inhibited cell proliferation of CNE2 cells compared with the control cells (Fig. 3b). EdU incorporation assays showed that overexpression of LET in CNE2 cells significantly decreased the ratio of EdU-positive cells (Fig. 3c). These results confirm that LET could inhibit cell proliferation. Next, we investigated whether the proliferation inhibition effect of LET is through apoptosis pathway. As shown in Fig. 3d, enhanced LET expression in CNE2 cells upregulated the level of cleaved caspase-3. TUNEL assays showed that overexpression of LET in CNE2 cells significantly increased the ratio of TUNEL-stained apoptotic cells (Fig. 3e). These results demonstrated that LET induces NPC cells apoptosis. To examine the effects of LET in NPC cell proliferation in vivo, we stably overexpressed LET in CNE2 cells, and subcutaneously injected the LET-overexpressed CNE2 and control CNE2 cells into nude mouse. CNE2 cells overexpressing LET showed significantly reduced tumor growth (Fig. 3f).

Repression of LET promotes cell proliferation and inhibits apoptosis of NPC cell

To further confirm the biological function of LET in NPC, we inhibited LET expression in HNE2 cells by transfecting

overall survival of 68 NPC patients. LET expression was detected by qRT-PCR. The median expression level was used as the cutoff, and each group has 34 cases

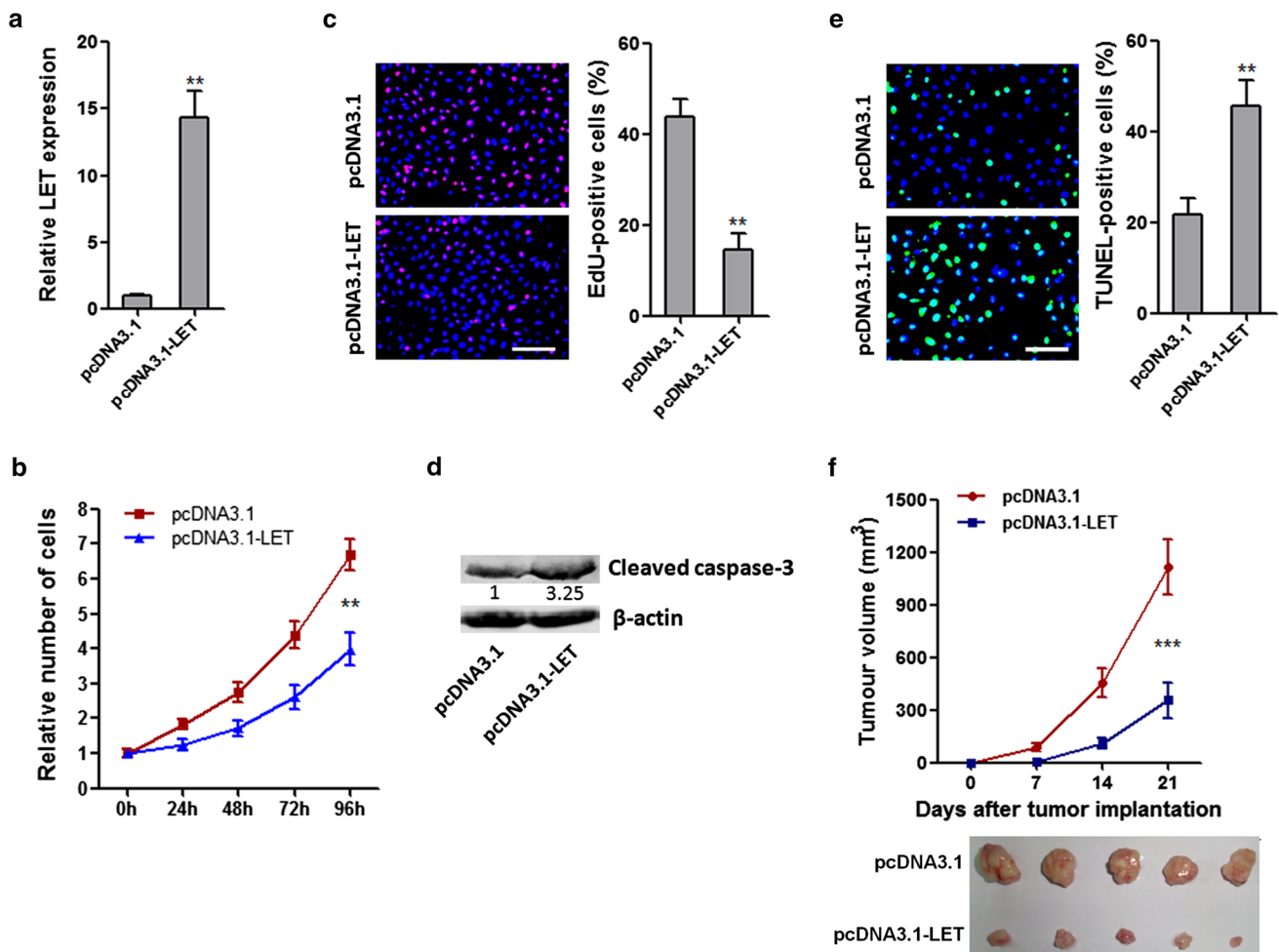


Fig. 3 LET inhibits NPC cell proliferation and induces apoptosis. **a** LET expression levels after transfection of the LET expression vector (pcDNA3.1-LET) or control vector into CNE2 cells. **b** The effects of LET on CNE2 cells proliferation were determined by the CCK-8 assay, and the relative number of cells to 0 h is presented. **c** The effects of LET on CNE2 cell proliferation were detected using EdU immunofluorescence staining. The blue color indicates the nuclei, and the red color represents EdU-positive nuclei. Scale bars

100 μm. *Right* percentages of EdU-positive cells. **d** The effects of LET on CNE2 cells apoptosis were determined by detecting cleaved caspase-3. **e** The effects of LET on CNE2 cells apoptosis were detected by TUNEL assay. Scale bars 100 μm. *Right* percentages of TUNEL-positive cells. For **a–e**, data are presented as the mean ± SE based on at least three independent experiments. ***P* < 0.01. **f** LET-overexpressed and control CNE2 cells were injected into nude mice subcutaneously, and tumor volume was determined. *n* = 5, ****P* < 0.001

LET-specific siRNAs (Fig. 4a). CCK-8 assays indicated that knockdown of LET significantly promoted cell proliferation of HNE2 cells compared with the control cells (Fig. 4b). EdU incorporation assays showed that knockdown of LET in HNE2 cells significantly increased the ratio of EdU-positive cells (Fig. 4c). These results suggest that knockdown of LET promotes cell proliferation. Knockdown of LET in HNE2 cells downregulated the level of cleaved caspase-3 (Fig. 4d). TUNEL assays showed that knockdown of LET in HNE2 cells significantly decreased the ratio of TUNEL-stained apoptotic cells (Fig. 4e). These data demonstrated that LET is involved in the progression of NPC through its effect on NPC cells proliferation and apoptosis.

LET is repressed by EZH2 and is inversely correlated with EZH2 levels in NPC tissues

We next sought to investigate the mechanism responsible for the downregulation of LET in NPC. We examined the promoter region of *LET* and identified consensus polycomb response elements (PRE) (Fig. 5a). Enhancer of zeste homolog 2 (EZH2), which induces histone H3K27me3 modification, is the catalytically active component of the polycomb response complex [25, 26]. In previous studies, EZH2 is shown to be upregulated in NPC tissues and promote NPC cells proliferation [27]. So we investigate whether EZH2 is responsible for the downregulation of LET in NPC. We performed ChIP assays to examine

whether EZH2 could bind the promoter region of *LET*. As shown in Fig. 5b, EZH2 immunoprecipitates were highly enriched in the promoter region of *LET* compared with control IgG immunoprecipitates. Next, we inhibited EZH2 expression in CNE2 cells by transfecting EZH2-specific siRNAs (Fig. 5c). ChIP assay results showed that knockdown of EZH2 reduced H3K27me3 level at the promoter region of *LET* (Fig. 5d). We further test whether EZH2 regulate the expression of *LET*. Our results showed that knockdown of EZH2 significantly increased *LET* expression in CNE2 cells (Fig. 5e). We also overexpressed EZH2 in CNE2 cells by transfecting pcDNA3.1-EZH2 (Fig. 5f), and the results showed that enhanced expression of EZH2 decreased *LET* expression (Fig. 5g). These data demonstrated that *LET* is repressed by EZH2-

Fig. 5 *LET* is repressed by EZH2-mediated histone H3 methylation. **a** Illustration of polycomb response element (PRE) within the *LET* promoter. **b** Products of ChIP using an EZH2 antibody were amplified by qPCR in CNE2 cells. Data in the *right panel* are expressed as a percent of input DNA. **c** EZH2 mRNA levels after the transfection of control siRNA or EZH2 siRNA into CNE2 cells. **d** Products of ChIP using an H3K27me3 antibody were amplified by qPCR in CNE2 cells after transfection with EZH2 siRNA for 48 h. Data in the *right panel* are expressed as a percent of input DNA. **e** *LET* transcript levels after the transfection of control siRNA or EZH2 siRNA into CNE2 cells. **f** EZH2 mRNA levels after the transfection of pcDNA3.1-EZH2 or pcDNA3.1 into CNE2 cells. **g** *LET* transcript levels after the transfection of pcDNA3.1-EZH2 or pcDNA3.1 into CNE2 cells. For **b–g**, data are presented as the mean \pm SE based on at least three independent experiments. ****** $P < 0.01$; ******* $P < 0.001$. **h** The correlation between EZH2 mRNA level and *LET* transcript level was measured in 68 NPC tissues. The respective expression values were subjected to Pearson correlation analysis ($r = -0.481$, $P < 0.001$, Pearson's correlation)

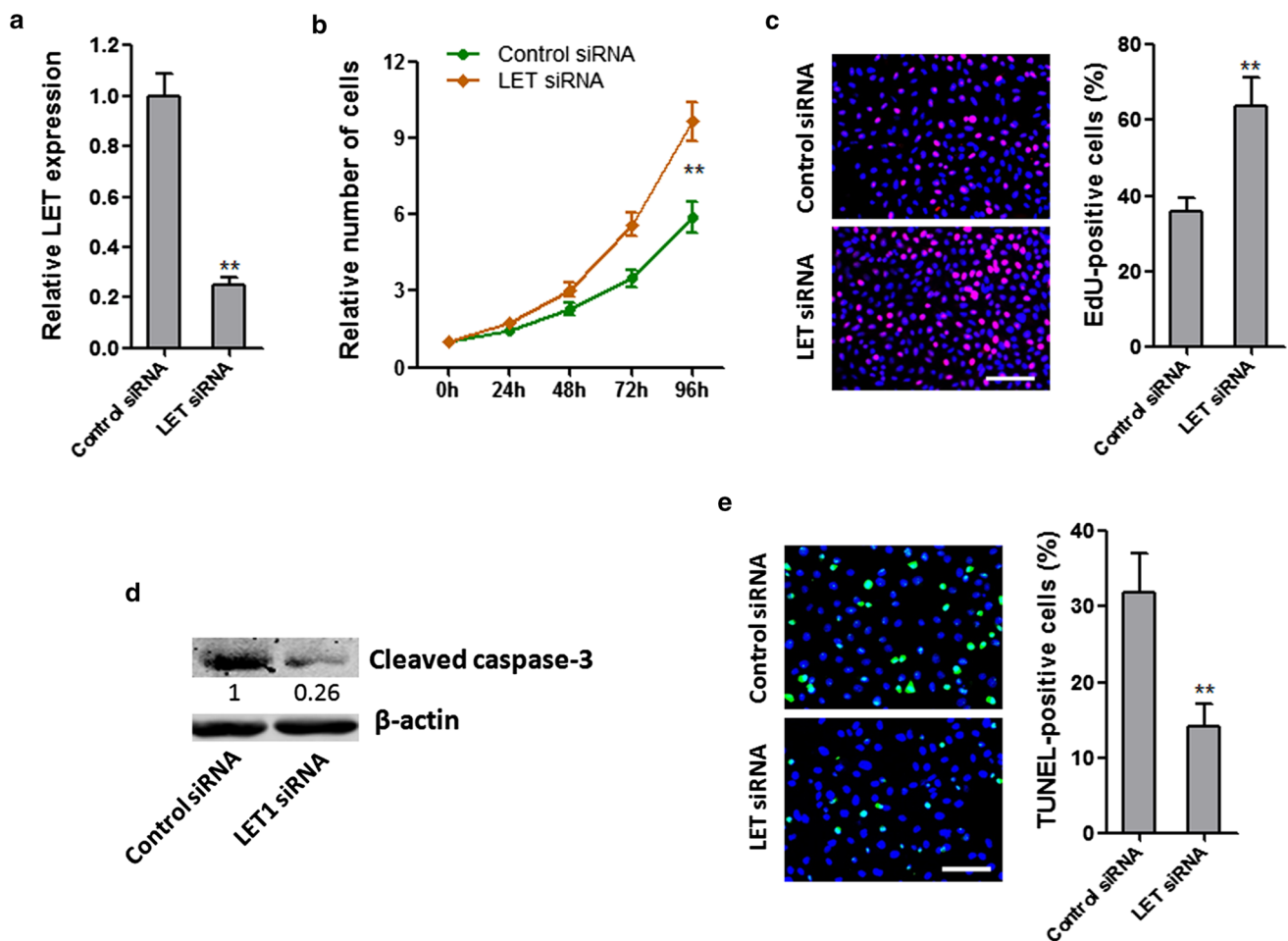
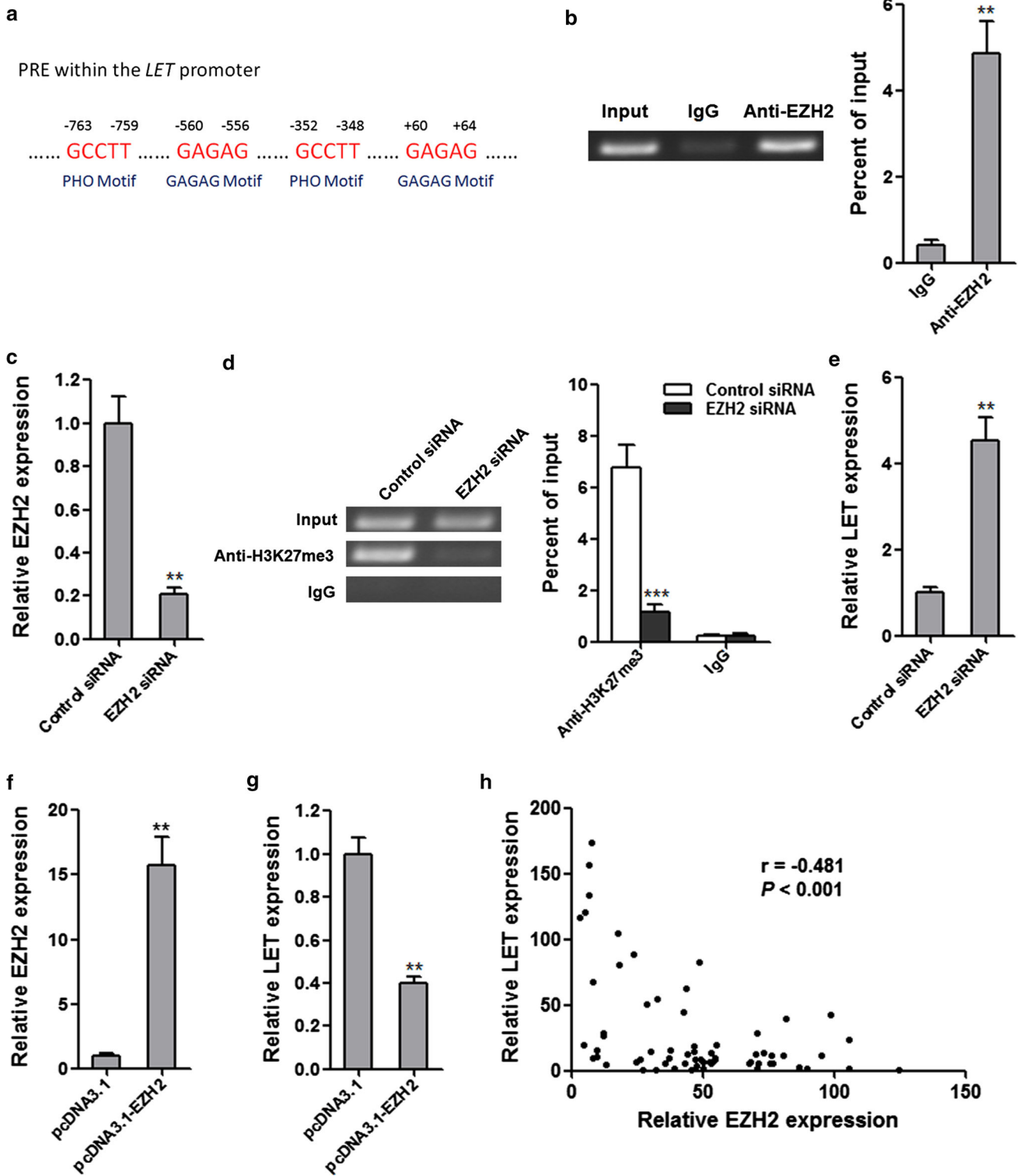


Fig. 4 Knockdown of *LET* promotes NPC cell proliferation and inhibits apoptosis. **a** *LET* expression levels after transfection of *LET* siRNA or control siRNA into HNE2 cells. **b** The effects of *LET* inhibition on HNE2 cells proliferation were detected by the CCK-8 assay, and the relative number of cells to 0 h is presented. **c** The effects of *LET* inhibition on HNE2 cell proliferation were detected using EdU immunofluorescence staining. The *blue color* indicates the nuclei, and the *red color* represents EdU-positive nuclei. *Scale bars*

100 μ m. *Right* percentages of EdU-positive cells. **d** The effects of *LET* inhibition on HNE2 cells apoptosis were determined by detecting cleaved caspase-3. **e** The effects of *LET* inhibition on HNE2 cells apoptosis were detected by TUNEL assay. *Scale bars* 100 μ m. *Right* percentages of TUNEL-positive cells. For all panels, data are presented as the mean \pm SE based on at least three independent experiments. ****** $P < 0.01$



mediated histone H3 methylation. Next, we examined whether LET is coexpressed with EZH2 in human NPC tissues. We measured the expression levels of EZH2 in the same set of 68 NPC tissues shown in Fig. 1 and then performed a correlation analysis of the EZH2 mRNA and

LET expression levels. A statistically significant inverse correlation was found between EZH2 mRNA and LET expression levels ($r = -0.481$, $P < 0.001$, Pearson's correlation, Fig. 5h), supporting the role of EZH2 in repressing LET.

Discussion

In this study, we examined the expression of long noncoding RNA-LET in NPC tissues and their adjacent normal tissues. We also detected the function of LET in NPC cells by applying gain-of-function and loss-of-function approaches. Our results demonstrate that LET is downregulated in NPC tissues in comparison with adjacent normal tissues, and that decreased LET expression is positively correlated with advanced clinical stage, larger tumor size, and increased lymph node tumor burden. Furthermore, we also found that low LET expression in NPC tissues indicates poor recurrence-free survival and overall survival of NPC patients. Enhanced LET expression inhibits cell proliferation and induces apoptosis of NPC cells, while the inhibition of LET promotes cell proliferation and inhibits apoptosis of NPC cells. Therefore, our results demonstrated that LET functions as a tumor suppressor in NPC.

The molecular mechanisms for the dysregulation of lncRNAs in cancer are not yet completely understood [9]. They can be regulated by cytokines and transcription factors, such as lncRNA-ATB activated by transforming growth factor beta, and lncRNA-CCAT1 and H19 induced by c-Myc [28–31]. Similar to mRNAs, lncRNAs could also be controlled by epigenetic regulation, including DNA methylation, histone acetylation, and histone methylation [32]. lncRNA-LET has been reported to be repressed by histone deacetylase 3-mediated histone deacetylation in hepatocellular carcinoma [21]. In this study, we further demonstrated that LET could also be regulated by histone methylation in NPC. Suppression of EZH2 induced a significant increase in LET expression, accompanied by a reduction of H3K27 methylation level at the promoter of *LET*. The inverse correlation between EZH2 mRNA and LET expression levels in NPC tissues supports EZH2's regulation of LET expression.

lncRNAs may exert their biological functions via regulating expression of cancer-associated genes in transcriptional level or post-transcriptional level [33–36]. However, LET has been reported to bind and downregulate nuclear factor 90 protein abundance in hepatocellular carcinoma [21]. The molecular mechanisms responsible for LET's function in NPC require further investigation.

In conclusion, our studies demonstrate that LET, which is repressed by EZH2-mediated histone H3K27 methylation, could regulate NPC cell proliferation and apoptosis, and is a prognosis factor in patients with NPC. Our findings indicate that LET is an important NPC biomarker for determining prognosis and a potential NPC therapy target.

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Compliance with ethical standards

Conflict of interest None.

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